

STUDIES ON PROGAGATION OF MICROBES
IN THE AIRBORNE STATE

3rd Quarterly Report, 1974-75

NASA Contract W13450

Task No. 193-58-62-13-10

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(NASA-CR-131844) STUDIES ON PROPAGATION
OF MICROBES IN THE AIRBORNE STATE
Quarterly Report, 1974 - 1975 (Naval
Biomedical Research Lab., Oakland) 16 p
HC \$3.25

N75-11590

Unclas

CSCI 06M G3/51 02254

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STUDIES ON PROPAGATION OF MICROBES IN THE AIRBORNE STATE

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ABSTRACT:

In attempts to demonstrate whether airborne microbes could propagate, we have (a) looked for dilution of a labelled base in DNA, (b) looked for labelling of DNA by mixing aerosols of the label and of the cells, (c) examined changes in cell size, (d) tested the possibility of spore germination, (e) and sought evidence of an increase in cell number.

All except (a) above have shown evidence that growth and propagation can occur under special conditions, principally at temperatures of 30 C (87 F) and water activity equivalents of 0.95 to 0.98. The next phase would be to delineate the limits of the phenomenon.

INTRODUCTION

The question of whether microbes can propagate (i.e., increase in number to such an extent that the species would exist indefinitely) in the atmosphere of Jupiter, regardless of whether the microbes originate from earth or whether they originate from evolutionary processes on the planet Jupiter, is the broader subject of this study. The present investigation asks whether it is possible for microbes to propagate in earth's atmosphere

For microbes to propagate on the planet Jupiter in a particulate form there are at least 6 processes that would have to be favorable. Listed without order of importance, they are:

(a) The particle must remain airborne in the altitude of biological significance (a shell approximately 80 km in thickness at pressures of 1 - 10 earth's atmosphere) for an interval greater than the division time of an airborne microbe (if that occurs).

(b) The particle must arrive and/or be in the atmosphere, regardless of whether it is an earth contaminant or the result of an evolutionary process. The former implies that an earth-borne microbe survived the restrictions of interplanetary travel and was ejected without harm into the proper atmospheric level.

(c) The microbe must be an anaerobe (i.e., oxygen cannot be the ultimate "energy sink" for metabolic processes that reduce energy-containing compounds to their lowest energy level), because there is no free oxygen on Jupiter. Earth's anaerobes can produce lactic acid or ethanol as "energy sinks"; Production of hydrocarbons might serve to form excess energy in a reducing atmosphere.

(d) The species must tolerate, and probably utilize, free ammonia as a nitrogen source. Some earth-borne species can, apparently, do this.

(e) There must be an abundance of particles that contain organic matter useful to the cell;

(f) There must be enough free water for biochemical process to function within the time-frame of a division cycle.

Because we had felt that it would be difficult, if not impossible, to demonstrate true propagation (cellular division) in the airborne state, our strategy was to attempt to first show that metabolism occurs, then formation of new DNA (genetic material), then growth in terms of increased cell mass, and finally formation of new cells. Data resulting from the successful completion of each phase would aid in experimental design for the next step.

Previous reports include data that demonstrate metabolism can occur. In our proposal, we indicated a number of other tests that would be conducted. The most feasible seemed to be an attempt to show dilution of a labelled base (^3H -Thymidine) incorporated into cells during the in vitro growth phase. Another test was to allow ^3H Thymidine particles to collide with airborne cells and look for incorporation of the base into DNA. Both of these approaches would test whether new DNA was formed. In these studies, humidity would be maintained at the highest practical level, and the Coulter electronic counter would be used to determine total cell numbers as well as changes in the size distribution. We also proposed to look at the possibility of spore germination, since it is known that, at high humidity, spores begin to lose their usual hardy state, and the death rate increases.

MATERIALS AND METHODS

Incorporation of ^3H -Thymidine in vitro

The species selected was Serratia marcescens.

For a successful experiment it was necessary to have (a) hardy cells, (a 15-hr culture was selected according to data from previous studies) (b) a high level of incorporation of thymidine and (c) a process that would remove

unincorporated thymidine without additional harm to the cells. The incorporation studies were conducted at the Biospherics Laboratories by Dr. Patricia Straat.

In brief, she found that the incorporation maximum of 25% occurs only if thymidine was added when the cell concentration was at an optical density value of 2.2 rather than at the 8-hr growth period indicated by initial tests (Fig. 1). Tests in our laboratory showed that the process devised by Dr. Straat did not appear to interfere with the usual behavior of 15-hr cells in the aerosol form.

Mixed aerosol studies

A. Thymidine and Bacteria

A solution of 1 Mci of ^3H -Thymidine in 7 ml with 2-Deoxy Adenosine (0.4%) was aerosolized from one atomizer and the bacteria (10 hr culture, 1.1×10^{10} cells/ml) was aerosolized from another. The two aerosols were mixed in air and conducted to a rotating drum as described in a previous report, except that the temperature was 30C and the relative humidity was 97-99%. Samples were taken at 30, 60 and 30 minutes aerosol time and tested for the presence of ^3H in the acid insoluble fraction as described by Dr. Straat in her report to NBRL.

B. Tryptone medium and bacteria

The tryptone medium was 1% tryptone 0.4% 2-Deoxy adenosine, and 1% glycerol in 0.1 molar phosphate buffer at pH 7.0. Bacteria (8 hr culture) were suspended in 7 ml of this medium after centrifugation and placed in one atomizer (1×10^{10} cells/ml); the second atomizer contained only the medium. The two fluids were aerosolized as in A, above.

Spore Studies

Stock preparations of spores of Bacillus subtilis, var niger had been frozen and stored at -30C. A preparation was opened, thawed, suspended in heart infusion broth heated to 80C for 10 minutes to kill any cells that might have germinated, and aerosolized into a stirred settling chamber (30C, 97% RH). At appropriate times, samples were taken by the impinger. Samples were assayed for viability before and after a heat shock (70⁰C for 10 minutes).

RESULTS

In an initial experiment with ³H-Thymidine incorporated into the cells, we found the amount incorporated to be only enough that by increasing sampling times, increasing the numbers of samplers per sample, and by atomizing for increased intervals, the level of sampled label would be suitable for semi-quantitative analysis of the data; we had expected to achieve a higher level, so further tests of this type were postponed.

In an initial experiment where a living bacterial aerosol was mixed with an aerosol containing ³H-Thymidine, we found about 8 times the amount of acid insoluble material had been formed compared to the amount formed in a similar test with dead cells. This is presumptive evidence of DNA formation, but the amount of activity in 25 liters of sampled air was, at most, 300 counts per minute, and marginal amounts of thymidine can attach to DNA fragments, thus becoming acid insoluble. Further work of this type will be pursued. However, data on survival of cells in this test indicated the occurrence of an increase in the numbers of viable cells above the number expected as a result of physical fall-out corrections and 100%

survival, but the increase was too small to be statistically convincing. Usually, "younger" cells (4-10 hr cultures) die more rapidly than "mature" cells (12-24 h cultures), but the possibility existed that in these special circumstances of high temperature, high humidity and a rich, hygroscopic medium, the reverse might obtain.

Accordingly, we tested an 8 hr culture, as above, except that no labelled thymidine was included. Results of the first test are shown in Fig. 2. The number of viable cells doubled over an interval of 360 minutes (7.5×10^5 to 1.4×10^6) and, when corrected for fallout, almost trebled (expected = 5×10^5).

In a second experiment (Fig. 3) where a simultaneous control test using no tryptone (but with live cells), was run, the number increased by about 18%, but when corrected for fallout the number had apparently doubled. Fig. 4 shows that cells not furnished tryptone did not increase in number in fact, the numbers of viable cells decreased at a more rapid rate than, the fallout, indicating death of some cells.

Whenever practicable, in aerosol experiments, samples were taken in an impinger containing 1% formalin to kill the cells, and the total number of cells (or insoluble particles) as well as the mass distribution was obtained. In general, the number of viable cells and the number of particles agreed within $\pm 10\%$, so we conclude that the particles we counted were, indeed, bacteria. Samples of air from drums that had been air-washed for 6 to 8 hrs (typical of the preparation for our aerobiological experiment) contained less than 0.01% of the particles present in the "zero" time sample of a usual test situation. Further, the mass distributions

of particles collected in the first samples from aerosols were identical to those obtained from either cells in the growth state at the time of aerosol production, or to cells in the atomizer fluid.

In all previous runs the mass distribution had tended to decrease in an almost linear manner with aerosol age, as shown in the control run, Fig. 5. In the presence of tryptone, however, the distribution decreased, then increased, then decreased (Fig. 6).

Results of the effect of high humidity on the ability of airborne spores to resist heat are shown in Fig. 7. In the figure, the percentage of heat sensitive cells into the total viable cells and spores collected as a function of aerosol time. The maximum, and subsequent decrease are caused, we believe, by germinating cells becoming sensitive to the air environment (thus being removed from the viable population), as well as to heat.

DISCUSSION

We believe these results, though unsupported by enough replicate data to provide an acceptable degree of quantitation, are evidence that bacteria can propagate in the airborne state under suitable circumstances. This conclusion is reached by the following:

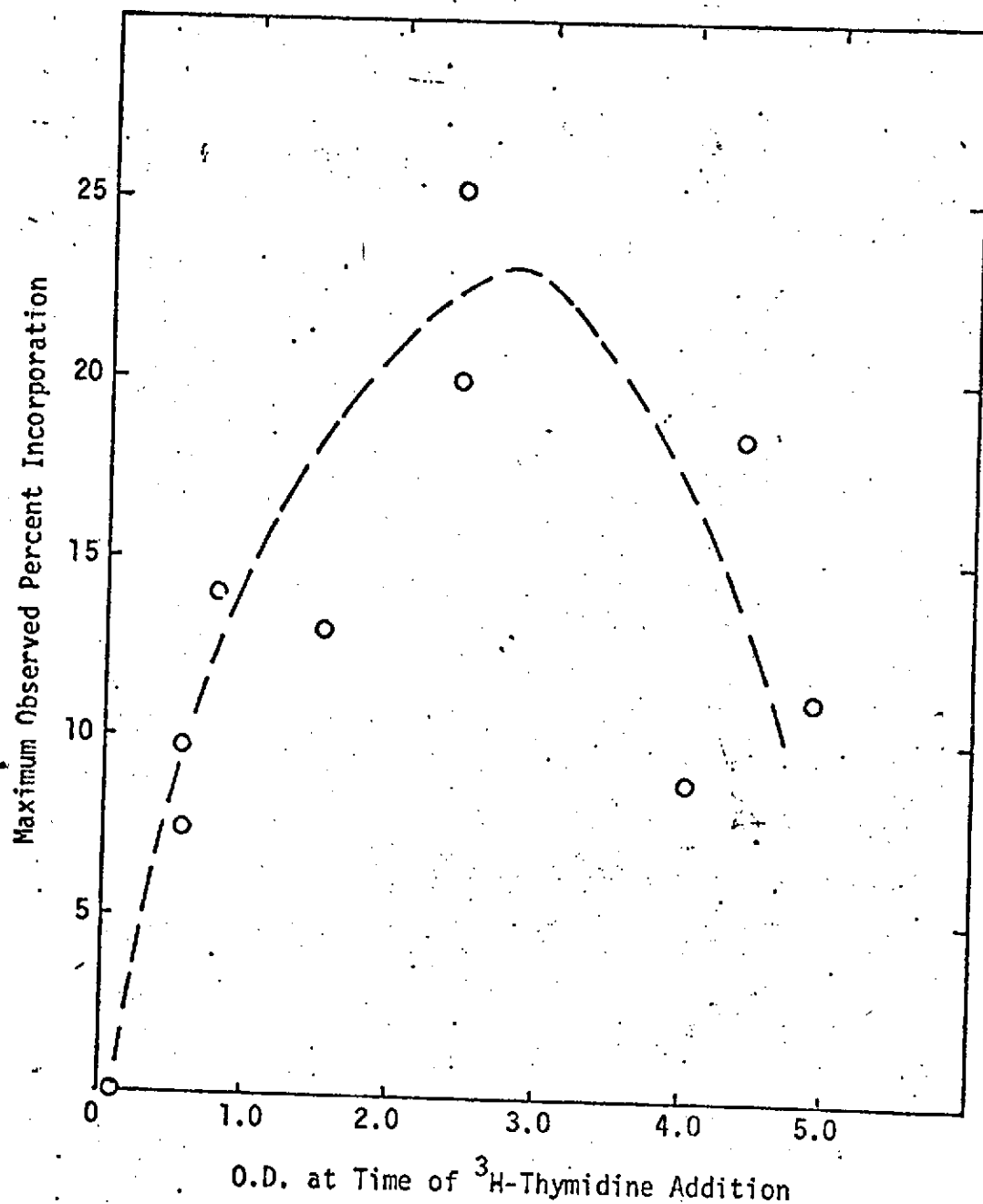
1. Airborne bacteria utilize glucose and form CO_2 , hence energy for growth is available.
2. A limited amount of ^3H -Thymidine appeared in the acid-soluble fraction of airborne cells exposed to particles of the labelled base; one explanation is that DNA was formed.
3. In two instances, airborne cells increased in viable numbers rather than decreasing as they would have by normal settling processes.

4. A similar increase (as in 3) in particles counted by the Coulter counter was observed.
5. The mass distribution of the airborne cells decreased, then increased, a process that must occur if division of cells also occurred.
6. Spores changed from a normal resting, resistive state, to one of sensitivity to heat; this is one step in the propagation of the species.

We will continue these tests, especially the use of labelled thymidine to measure DNA formation, until either the evidence is shown not to be artificial or until some non-growth-related process can be shown to have influenced the results.

Notwithstanding the fact that propagation of airborne cells apparently occurs at least in a limited circumstance, these data show that vegetative cells, and probably spores, have adaptive systems that do support such a process. We do not know the boundaries, however, of temperature, humidity, medium, age of culture, atmospheric content, anaerobiosis etc., within which the process can operate, nor do we know how these factors would effect the time-constant (about 130 minutes in these experiments; see Fig. 6). The latter is important because our studies have been limited to 24 hrs; we would not have observed propagation that might have occurred over longer periods. The question is also related to the latest estimate of the time-constant for atmospheric turbulence on Jupiter-about 2 month--in which particles could be "trapped" for years.

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Incorporation as a Function of Culture Optical Density

At various times after inoculation, 2-deoxyadenosine and ³H-thymidine were added to *Serratia* cultures. The resulting incorporation of tritium into acid insoluble material was determined as a function of time after the addition (Tables I and II). The maximum incorporation attained is expressed as a function of optical density at 420 mμ of the culture at the time of the ³H-thymidine addition.

Figure 1.

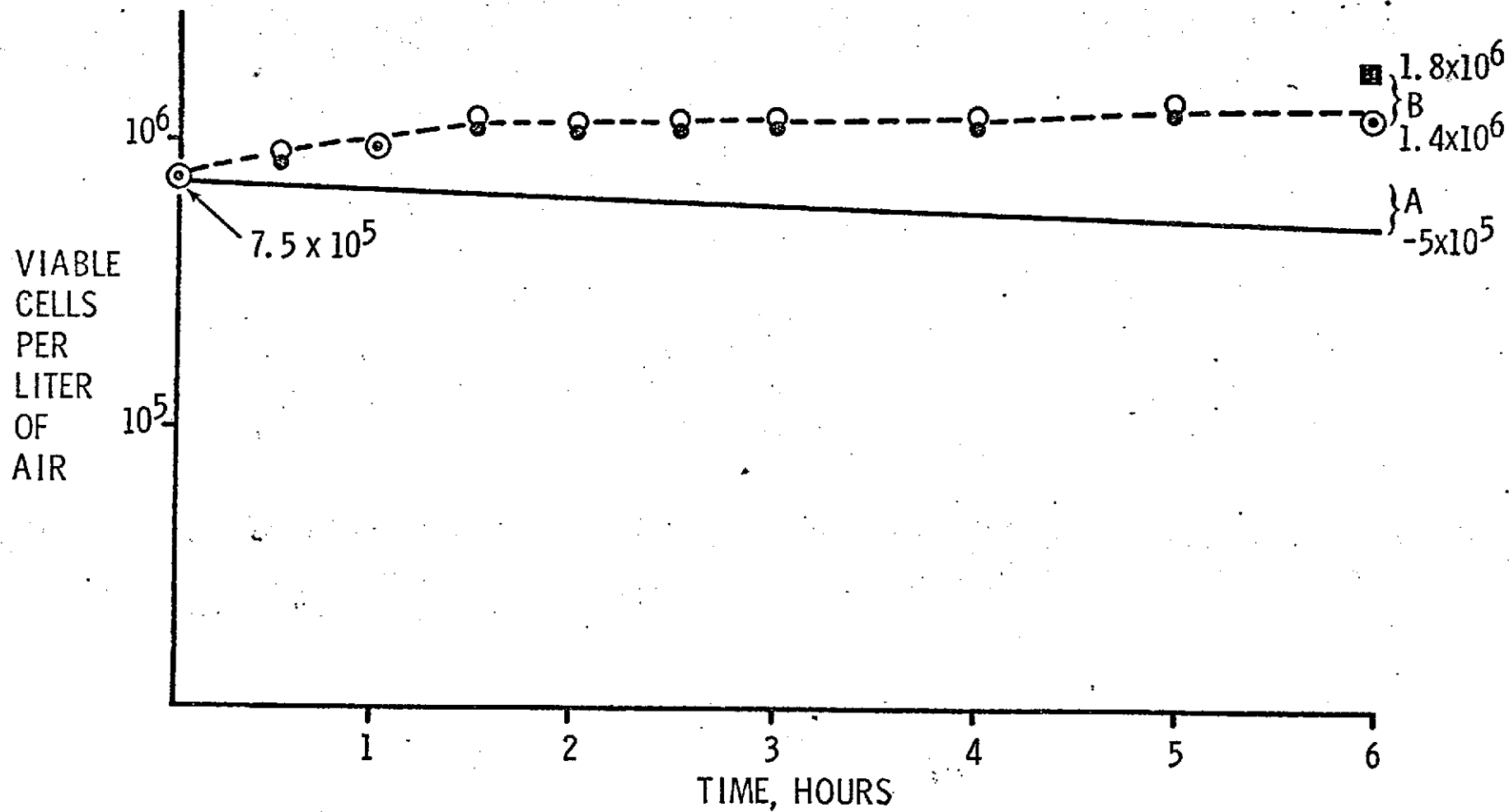


Fig. 2. Viable cells (●) and cell number (○) in a mixed aerosol with tryptone. Solid line is particle decay.

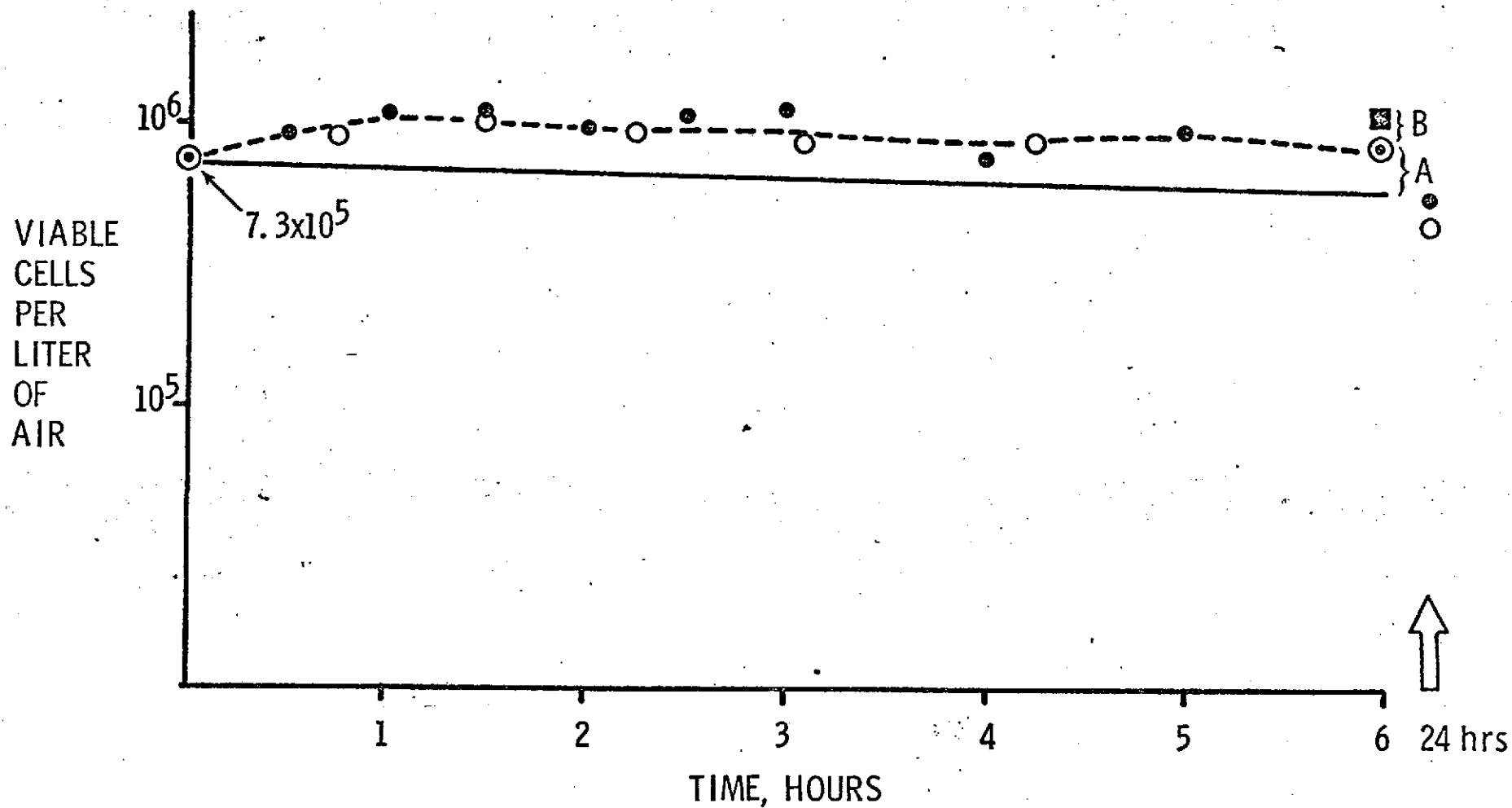


Fig. 3. Viable cells (●) and cell number (○) in a mixed aerosol with tryptone. Solid line is particle decay.

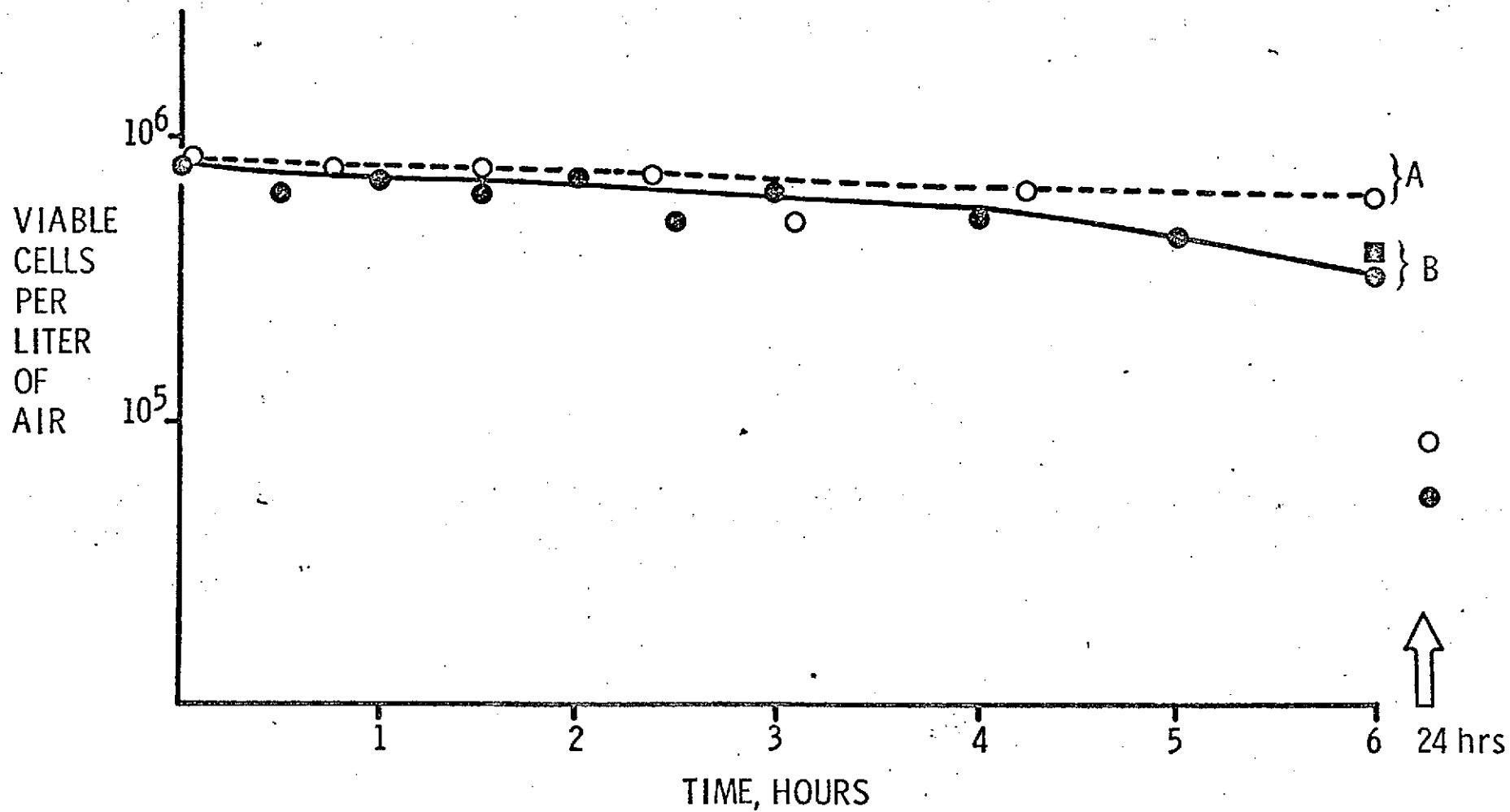


Fig. 4. Viable cells (●) and cell number (○) in a mixed aerosol without tryptone. Solid line is particle decay.

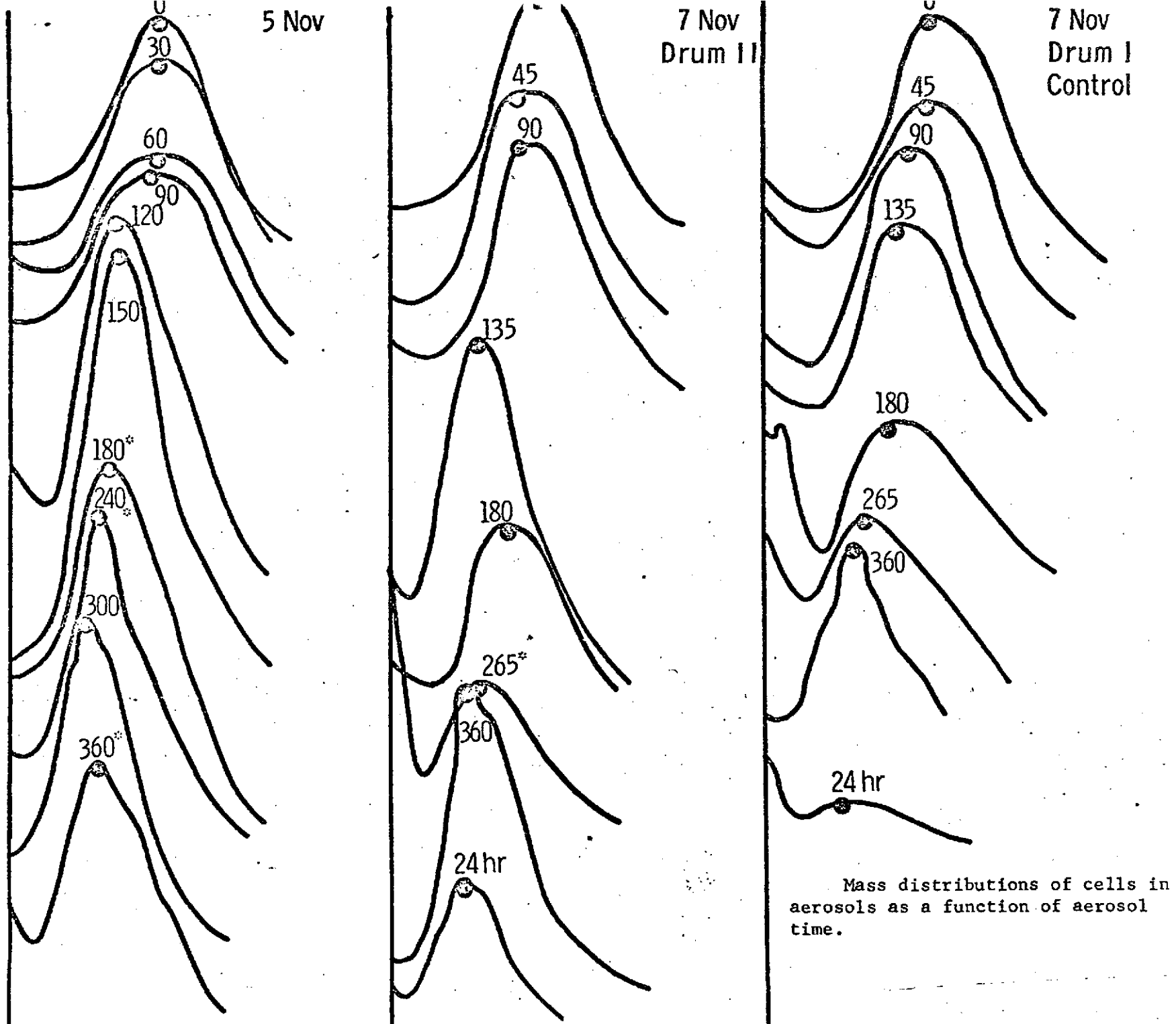


Figure 5.

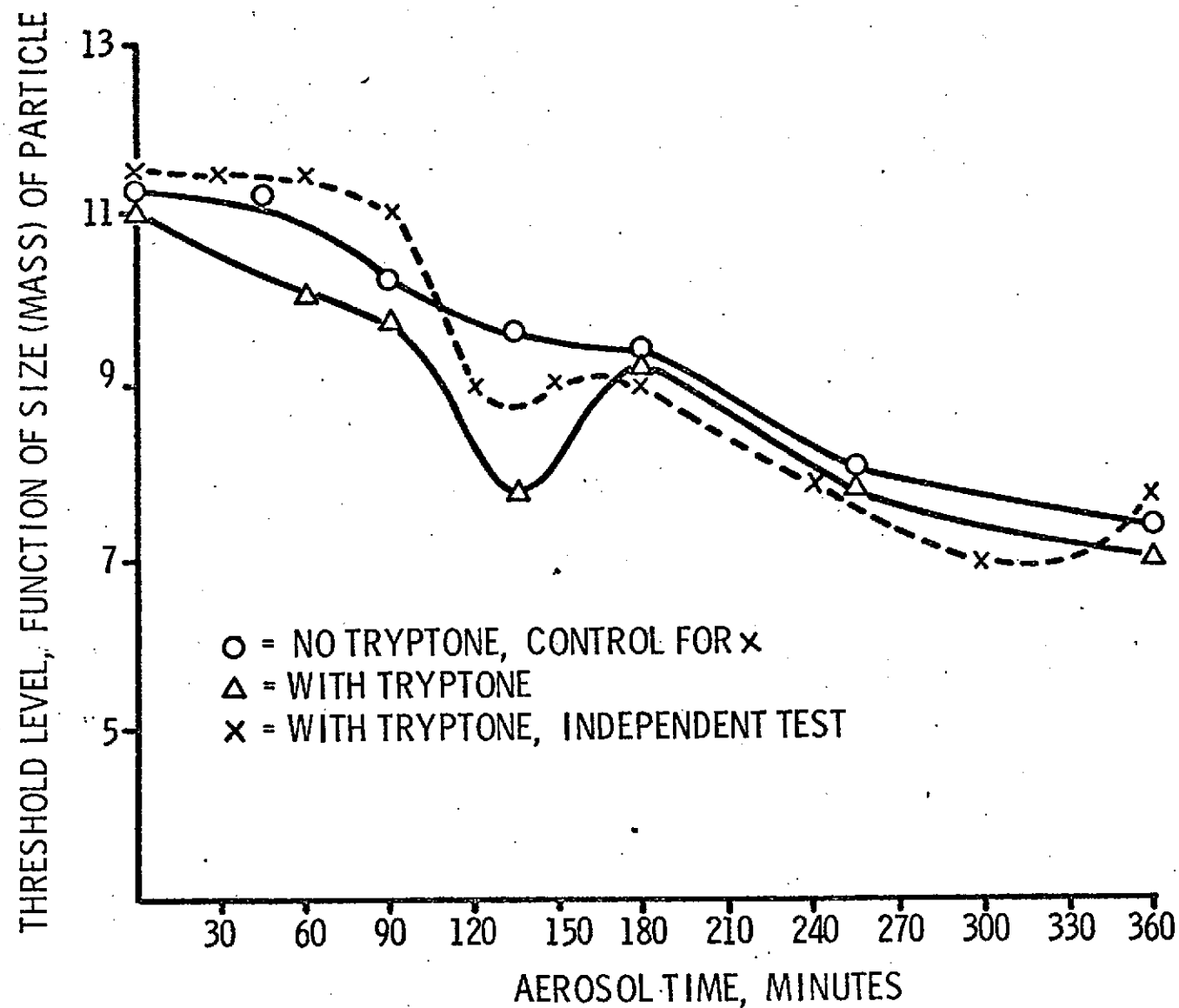


FIG. 6.

Change in the mode of the mass (size) distribution of airborne cells with aerosol time.

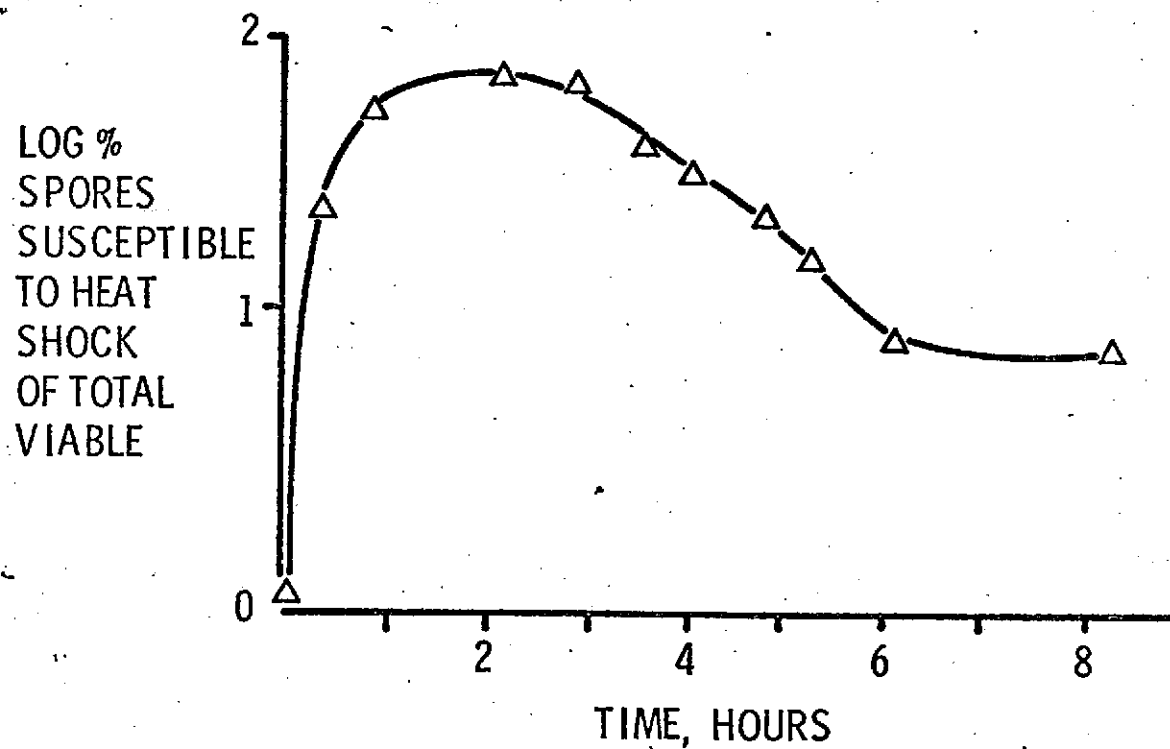


Fig. 7.

Change in heat susceptibility of spores of Bacillus subtilis with aerosol age.